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Research Note

Lack of Effect of Feeding Citrus By-Products in Reducing *Salmonella* in Experimentally Infected Weanling Pigs†

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ABSTRACT

The objective of the current research was to determine if feeding the citrus by-products D-limonene (DL) and citrus molasses would reduce the concentration and prevalence of *Salmonella* in weanling pigs experimentally infected with *Salmonella* Typhimurium. Twenty crossbred weanling pigs (average body weight [BW], 19.9 kg) were randomly assigned to one of four treatments: control, low-dose DL (1.5 ml/kg of BW per day), high-dose DL (3.0 ml/kg of BW per day), and citrus molasses (0.05 kg/kg of BW per day). Treatments were administered in the feed (twice daily) for 7 days, with one-half of the dose administered at each feeding. Fecal samples were collected twice daily (prior to administration of treatment) and cultured for quantitative and qualitative determination of the challenge strain of *Salmonella*. Upon termination of the study, pigs were euthanized and tissues from the stomach, ileum, cecum, spiral colon, and rectum, as well as luminal contents, were collected. In addition, the popliteal and ileocecal lymph nodes and liver, spleen, and tonsil tissue were collected for qualitative *Salmonella* culture. No significant treatment differences ($P > 0.05$) were observed among treatments for fecal concentration or prevalence of *Salmonella* throughout the 7-day collection period. Likewise, no treatment differences ($P > 0.05$) were observed for any of the tissue or luminal content samples collected. *Salmonella* was not cultured from the muscle-bound popliteal lymph node but was cultured from the mesenteric ileocecal lymph nodes. While there were no effects in the current experiment, future research may examine the effect of a lower challenge dose and/or different administration (dose or duration) of the citrus by-products.

Salmonella enterica infections result in an estimated 1.4 million human cases and approximately 500 deaths annually in the United States *(5, 8)*. Approximately 95% of the human cases of salmonellosis have been linked to the consumption of contaminated products such as beef, pork, poultry, eggs, milk, seafood, and fresh produce *(5)*. *Salmonella* in swine populations continues to present herd health challenges throughout the pork production chain, as well as food safety issues at harvest. Recognizing that swine are known reservoirs for *Salmonella,* farm managers and public health officials alike have sought efficacious control strategies. Slaughter and processing interventions have made significant strides in controlling *Salmonella*; however, contamination of pork products still persists. In a national study, 9.6% of retail samples, including freshly ground pork and whole-muscle store-packaged products, were culture positive for *Salmonella (4)*. Naturally, implementation of preharvest mitigation tools to control and reduce *Salmonella* on farms has garnered increased attention.

Essential oils derived as by-products of the citrus industry have been investigated for antimicrobial properties against common foodborne bacteria and have been shown to possess bactericidal properties *(3)*. Novel research investigating the effects of the essential oil D-limonene (DL) and citric acid on *Salmonella* in chicken crops demonstrated beneficial effects under the study conditions *(1)*. We hypothesized that similar results may also be achieved in other monogastric species, such as swine. Therefore, the objective of the current research was to determine if feeding either DL or citrus molasses (MOL) would reduce *Salmonella* populations in experimentally infected pigs.

MATERIALS AND METHODS

Twenty crossbred commercial weanling pigs (average body weight [BW], 19.9 kg) were purchased locally and transported to our laboratory where they were housed indoors in individual pens and randomly assigned to one of four treatments. Piglets were maintained on the same diet as they were fed prior to purchase, a commercially available, 16% protein, swine grower ration medicated with chlortetracycline (100 g/ton) formulated to meet or exceed the nutrient requirements for pigs this age *(6)*. All procedures involving animals and their care were preapproved and monitored by the Animal Care and Use Committee of the Southern Plains Agricultural Research Center, Agricultural Research Service–U.S. Department of Agriculture, College Station, TX. Following a 2-week adaptation period, piglets were randomly

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assigned to one of the treatments: control (CON; corn oil only), low-dose DL (LDDL; 1.5 ml of DL per kg of BW per day), high-dose DL (HDDL; 3.0 ml of DL per kg of BW per day), and MOL (0.05 kg of MOL per kg of BW per day). Treatments were administered twice daily (0700 and 1400 h) for 7 days via mixing with 100 g of their daily feed. Once the treated feed was consumed, the pigs were given one-half of their total remaining feed allotment for the day. DL (95.5%) and MOL, a by-product of the citrus industry consisting largely of citrus peels, sugars, organic acids, and phenolic compounds, were kindly provided by the Texas Citrus Exchange in Mission. One day prior to initiation of the treatments, pigs were experimentally infected via oral gavage with 6 ml of tryptic soy broth containing 7.5×10^8 CFU/ml *Salmonella* Typhimurium. This isolate was obtained from the National Veterinary Service Laboratories, Ames, IA, and made resistant to nalidixic acid (20 μg/ml), novobiocin (25 μg/ml), and chlortetracycline (30 μg/ml) via successive transfers in antibiotic-containing broth. Prior to the initiation of the experiment, fecal samples were collected from all pigs and prescreened to ensure that no animals were shedding wild-type *Salmonella* in their feces capable of growth on brilliant green agar containing the antibiotics described below. Fecal samples were collected twice daily for qualitative and quantitative determination of *Salmonella* throughout the 7-day treatment period. Upon study termination, all pigs were euthanized (Euthasol euthanasia solution, Delmarva Laboratories, Inc., Midlothian, VA) and tissues from the stomach, ileum, cecum, spiral colon, and rectum, as well as their respective contents, were aseptically collected for qualitative and quantitative (contents only) culture of the challenge strain of *Salmonella*. Additional tissue samples from the liver, spleen, and tonsil, as well as the ileocecal and popliteal lymph nodes, were collected for culture as described below.

Bacterial culture and enumeration. Tissue and luminal contents were qualitatively cultured by enriching approximately 10 g (±0.1 g) of each sample in 90 ml of tetrathionate broth (37°C for 24 h), followed by a second enrichment in Rapport-Vassilidis broth (100 μl in 5 ml at 42°C for 24 h). The second enrichment was plated on brilliant green agar (Oxoid Ltd., Hampshire, UK) supplemented with nalidixic acid (20 μg/ml), novobiocin (25 μg/ml), and chlortetracycline (30 μg/ml) and incubated (37°C for 24 h). Following incubation, colonies exhibiting typical *Salmonella* morphology were confirmed biochemically using lysine and triple sugar iron agars. For quantitative determination, 100 μl of the feces–tetrathionate broth mixture (prior to incubation) was spiral plated onto xylose lysine desoxycholate (Oxoid Ltd., Hampshire, UK) agar supplemented with nalidixic acid (20 μg/ml), novobiocin (25 μg/ml), and chlortetracycline (30 μg/ml) using a commercially available spiral plater (Spiral Biotech Autoplate 4000, Advanced Instruments, Inc., Norwood, MA). Plates were incubated overnight at 37°C. Black colonies exhibiting typical *Salmonella* morphology were counted, and *Salmonella* populations calculated and converted to CFU (log) per gram of feces or luminal contents.

Statistical analysis. Data were analyzed using SAS version 9.02 (SAS Institute Inc., Cary, NC). Data for qualitative (positive or negative) and quantitative fecal shedding, as well as qualitative tissue and luminal content culture, were analyzed using the Proc Glimmix procedure, and contrast statements developed to compare the results for LDDL, HDDL, and MOL treatments to those of the CON treatment. As animals were treated and maintained individually, individual pigs were used as the experimental unit in each of the statistical analyses. Differences among treatments were considered significant at a 5% level of significance.

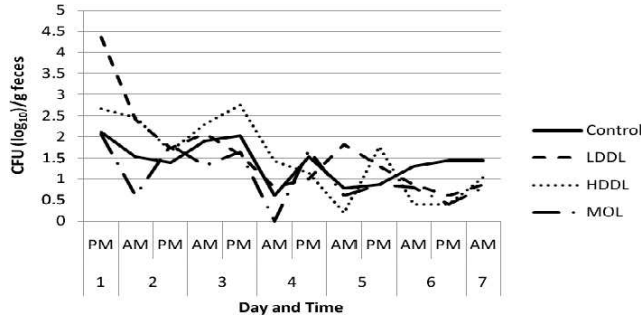

FIGURE 1. *Mean CFU (log) of* Salmonella *per gram of feces by sampling time periods and treatment in experimentally infected pigs administered either corn oil (control), 1.5 ml of* D*-limonene per kg of BW per day (LDDL), 3.0 ml of* D*-limonene per kg of BW per day (HDDL), or 0.05 kg of citrus molasses per kg of BW per day (MOL) in their feed. No significant differences were observed between control and treated animals (*$P > 0.05$*).*

RESULTS

Prior to inoculation with *Salmonella* Typhimurium, fecal samples representing all pigs were culture negative for ''wild-type'' *Salmonella* populations capable of growth on antibiotic-supplemented agar (data not shown). No treatment differences were observed ($P = 0.18$) for the enriched fecal samples throughout the 7-day collection period following experimental infection (Fig. 1), and no significant differences were observed when the data for each treatment were compared with those for control animals (CON versus LDDL, $P = 0.30$, and CON versus HDDL, $P = 0.59$). For the MOL treatment group, there was a tendency ($P = 0.08$) for fewer pigs to shed *Salmonella* following enrichment of fecal samples than for pigs receiving the CON treatment. No treatment differences were observed ($P > 0.05$) in the percentages of *Salmonella*-positive tissues collected at necropsy. The majority of tonsil, cecum, and spiral colon tissue samples were positive for *Salmonella* (90, 80, and 65%, respectively). No popliteal lymph nodes were culture positive for the challenge *Salmonella* strain. Luminal content samples cultured quantitatively and qualitatively were likewise not different among treatments ($P > 0.05$) (Table 1).

DISCUSSION

The results of the current research failed to demonstrate a beneficial effect of DL or MOL in reducing *Salmonella* fecal shedding or the prevalence or concentration of *Salmonella* in the gastrointestinal tissue and luminal contents in experimentally infected pigs. Quite possibly the challenge dose used in this study simply overwhelmed any protective effect that may have been exerted by the citrus product treatments. Previous research in our laboratory has found that a higher challenge dose, such as used in this experiment, is required to maintain fecal shedding and recover the inoculated strain in intestinal tissue and contents several days after infection. Granted, utilizing a lower dose more similar to what pigs might be exposed to under normal production parameters would be less likely to overwhelm any benefits of the treatment. However, due to the difficulty

TABLE 1. *CFU of* Salmonella *and percentages of positive samples in luminal contents and tissues of experimentally infected pigs treated with* D*-limonene or citrus molasses*[a]

Sample type and measure	Treatment			
	CON	LDDL	HDDL	MOL
Luminal contents				
Concn (log CFU/g)				
Stomach	0.7	0	0	0
Ileum	1.1	0.5	0.6	0.8
Cecum	1.3	3.5	3.4	1.9
Spiral colon	2.2	3.8	3.4	2.6
Rectum	1.9	1	0	1.9
% positive with enrichment				
Stomach	20	0	0	0
Ileum	40	20	20	20
Spiral colon	60	100	100	80
Cecum	60	100	100	40
Rectum	60	40	0	40
Tissues				
% positive with enrichment				
Stomach	20	0	20	0
Ileum	20	0	20	40
Spiral colon	60	60	60	80
Cecum	80	100	60	80
Rectum	40	0	20	40
Ileocecal lymph nodes	20	0	20	20
Popliteal lymph node	0	0	0	100
Spleen	20	0	0	20
Tonsil	80	100	80	0
Liver	0	20	0	0

[a] Data are the mean CFU (log) of *Salmonella* per gram in luminal content samples and percentages of *Salmonella*-positive luminal contents and tissue samples collected throughout the gastrointestinal tract of experimentally infected pigs administered corn oil (control [CON]), 1.5 ml of D-limonene per kg of BW per day (LDDL), 3.0 ml of D-limonene per kg of BW per day (HDDL), or 0.05 kg of citrus molasses per kg of BW per day (MOL) in their feed. No significant differences were observed between control and treated animals ($P > 0.05$).

in recovering a challenge strain administered at these lower infectious doses, the number of animals utilized would need to be increased substantially to detect the probably more subtle differences. As adding more animals was not a realistic option due to financial constraints, we utilized the higher challenge dose.

Perhaps increasing the amount of citrus product would have provided for a significant reduction in the *Salmonella* present in these pigs. Although this is certainly possible, we were concerned that increasing the amount of DL administered in the feed might result in reduced palatability and feed intake issues and/or irritate the mucosal surface of the oral cavity and gastrointestinal tract. The MOL, while relatively palatable, was found to inhibit feed intake when incorporated at levels any higher than what was utilized in this research (unpublished data). Therefore, the only alternative would be to increase the duration of feeding the citrus by-products. In hindsight, it may have proved beneficial to start the administration of treatments prior to *Salmonella* challenge and/or feed for longer than 7 days postinfection. While the former does not appear to have any drawbacks and makes good sense, the latter could potentially reduce the number of *Salmonella*-positive samples recovered due to the slow ''washout'' of the challenge strain in this type of study.

Our hypothesis, based on the reported beneficial effects of citrus by-products on *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in sheep and the antimicrobial properties of DL *(2, 7),* was that we could reduce the concentration of *Salmonella* with the administration of DL and MOL. Unfortunately, we did not observe any beneficial effects of the citrus products under these experimental conditions. Worth mentioning is the finding that the muscle-bound popliteal lymph nodes collected during necropsy were all culture negative for *Salmonella,* as these and other muscle-bound lymph nodes could enter the food chain via ground-meat products. While our findings do not support the use of citrus by-products to reduce the *Salmonella* burden in swine, further research may be warranted using modifications of the experimental procedures discussed above.

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